Journal of FISH BIOLOGY
Published for the Fisheries Society of the British Isles

Journal of Fish Biology (2013) **82**, 637–657
doi:10.1111/jfb.12022, available online at wileyonlinelibrary.com

Genetic differentiation and hybridization in two naturally occurring sympatric trout *Salmo* spp. forms from a small karstic lake

P. Gratton*†, G. Allegrucci†, A. Gandolfi*‡ and V. Sbordoni†

**Department of Biodiversity and Molecular Ecology, Research and Innovation Centre, Fondazione Edmund Mach, Via E. Mach 1, I-38010 San Michele all'Adige (TN), Italy and †Department of Biology, University of Rome "Tor Vergata", Via della ricerca scientifica snc, I-00173 Roma, Italy*

(Received 30 December 2011, Accepted 6 November 2012)

In this study, multiple molecular markers [genotyping of 12 nuclear microsatellite loci and the protein-coding gene *ldh-c1* * plus sequencing of the mitochondrial DNA (mtDNA) control region] were employed to investigate the genetic structure of the two trout forms, *Salmo cettii* and *Salmo fibreni*, inhabiting Lake Posta Fibreno, central Italy. The two forms were found to share a unique mtDNA haplotype, belonging to a widespread Mediterranean haplogroup (AD). Bayesian clustering analyses showed that these two forms correspond to well-defined autochthonous gene pools. Genetic introgression between the two gene pools, however, was observed, whose frequency appears to correlate with the environmental features of the spawning sites. The interplay of selection for the spawning sites, philopatry and natural selection can be argued to maintain genetic differentiation despite the lack of complete reproductive isolation.
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Key words: genetic structure; introgression; Lake Posta Fibreno; macrostigma; *Salmo cettii*; *Salmo fibreni*.

INTRODUCTION

Salmonids exploit available ecological space by means of an extraordinary phenotypic variation that originates by a complex interplay of phenotypic plasticity, genetic divergence and hybridization (Hutchings, 2011). Thus, salmonids represent valuable model systems for the study of evolutionary processes (Stearns & Hendry, 2004). Geographically separated populations exposed to different selective pressures have been shown to undergo local adaptation (Fraser *et al*., 2011) and many instances of partial or complete reproductive isolation among phenotypes occurring in sympatry have been reported (Ferguson & Mason, 1981; Sell & Spirkovski, 2004; Sušnik *et al*., 2007; Bernatchez *et al*., 2010).

Therefore, both natural selection and limited gene flow (due to geographical, *i.e*. allopatric differentiation, and ecological, *i.e*. sympatric differentiation, barriers) play a decisive role in the evolution and maintenance of genetic variation.

‡Author to whom correspondence should be addressed. Tel.: +390461615532; email: andrea.gandolfi@fmach.it

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In turn, differentiation between populations at genetically determined adaptive traits sets the conditions for the evolution of intrinsic reproductive barriers and, ultimately, speciation (Bernatchez *et al*., 2010). On the other hand, even ecologically and phylogenetically distant evolutionary units, within different salmonid genera, have been proven to hybridize extensively (Leary *et al*., 1995; Kahilainen *et al*., 2011).

As a consequence of the complexity of evolutionary process in salmonids, the taxonomy of this group is still very unstable. Several described taxa were defined on the basis of morphological differences and geographic isolation and still await molecular assessment of their genetic identity (Kottelat & Freyhof, 2007; Jonsson & Jonsson, 2011).

The Mediterranean Sea basin harbours the largest share of the taxonomic, ecologic and phylogenetic diversity in the *Salmo trutta* L. 1758 complex (Kottelat & Freyhof, 2007). A significant portion of this genetic heritage has been lost, however, owing to environmental degradation, overfishing and stocking of allochthonous strains (Poteaux *et al*., 1999; Berrebi *et al*., 2000). Anthropogenic pressure has been especially intense in the Italian peninsula. Most residual Italian *Salmo* spp. populations, in fact, display strong introgression due to massive stocking of non-native *S. trutta* (Gandolfi *et al*., 1991; Nonnis Marzano *et al*., 2003; Caputo *et al*., 2004).

Two different forms of salmonids have been recognized for centuries in Lake Posta Fibreno (central Italy) and its tributaries (Salviani, 1554; Carbone, 1965). Zerunian & Gandolfi (1986) reviewed the variation in external morphology (mostly size and colour pattern) of Posta Fibreno *Salmo* spp. and identified two reference autochthonous form types, which they referred to as macrostigma [until recently, autochthonous *S. trutta* in the Tyrrhenian basin were referred to as *Salmo trutta macrostigma* (Duméril 1858), Gandolfi *et al*., 1991] and fibreni. A third form (leopardo) was also described, scarcely different from macrostigma (essentially showing a higher number of dark spots on the flanks). The last type was tentatively interpreted as a possible hybrid between the autochthonous macrostigma and an allochthonous fario type (hatchery *S. trutta*). The leopardo form has not been mentioned in later literature (Gandolfi *et al*., 1991).

Further investigation led the same authors to classify the fibreni form as a new species of *Salmo*, endemic to the Posta Fibreno basin: *Salmo fibreni* Zerunian & Gandolfi 1990 (Zerunian & Gandolfi, 1990). The description of the new species was based on the significant differences in meristic characters (number of vertebrae) between samples of fibreni and macrostigma visually assigned *a priori* (Zerunian & Gandolfi, 1990). The most significant difference between the two forms, however, is probably the size at sexual maturity; Zerunian & Gandolfi (1990) reported that all fibreni males >12 cm total length (L_T) and all females >14 cm L_T were sexually mature, while the corresponding figures for macrostigma were reported by Gibertini *et al*. (1990) at 17–19 cm and 28–30 cm L_T, respectively. Fibreni individuals >23 cm L_T were never reported, so that Gandolfi *et al*. (1991) hypothesized that the fibreni form attains sexual maturity at the end of its first year and very rarely survives through the second and third years. At present, the distinctive colour patterns (Zerunian & Gandolfi, 1986, 1990; Gandolfi *et al*., 1991) are, along with the size of mature individuals, *de facto* practical standards to identify the two forms for management-related and conservation-related activities (Fig. 1). Recent taxonomical syntheses (Kottelat & Freyhof, 2007) retain *S. fibreni* as a valid taxon, and consider *Salmo cettii* Rafinesque 1810 to be the valid name for all other autochthonous

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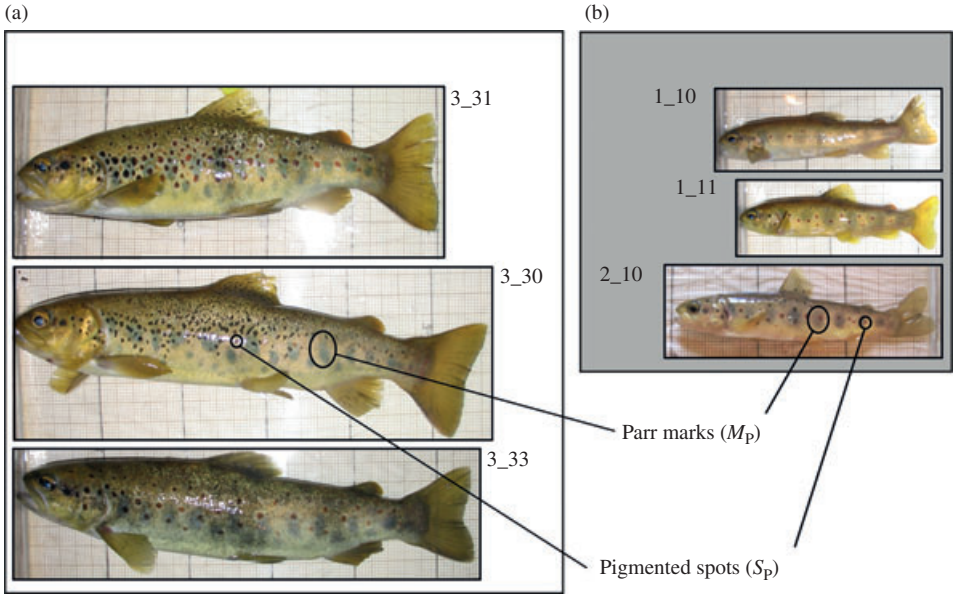

FIG. 1. Representative adult individuals of the (a) macrostigma and (b) fibreni phenotypes. Fishes were photographed prior to release on a mm scale. All photographs are presented at approximately the same scale. Colour pattern features considered in the analyses are highlighted. Individual codes beside each photograph refer to sampling site and individual identification.

Salmo spp. in the Tyrrhenian basin. The phenotypic characters given by Kottelat & Freyhof (2007) for *S. cettii* are essentially identical to (and largely based on) the description of the macrostigma (and leopardo) form from Lake Posta Fibreno given by Zerunian & Gandolfi (1986) and Gandolfi *et al*. (1991). Therefore, in the interest of consistency with previous literature, the name macrostigma will be used to refer to the phenotypic form described by Zerunian & Gandolfi (1986) and Gandolfi *et al*. (1991).

Both the macrostigma and fibreni forms can, at least occasionally, be found throughout the lake and its tributaries. Fibreni individuals, however, are most frequently captured and seen along the eastern shores of the lake (Zerunian & Gandolfi, 1986, 1990; Gandolfi *et al*., 1991; D'Orsi & Seminara, 2010). Spawning of fibreni individuals has been reported at karstic springs, either flowing directly into the lake or feeding the south-eastern tributary Torrente Dova (Zerunian & Gandolfi, 1986, 1990; Gandolfi *et al*., 1991; D'Orsi & Seminara, 2010) (Fig. 2). According to Gibertini *et al*. (1990), spawning of the fibreni form peaks from December to January, although females with mature gonads have been observed from October to April (Zerunian & Gandolfi, 1990; Zerunian *et al*., 1994). On the other hand, macrostigma spawning is known to occur from late December to February in streams and rivers of the Posta Fibreno basin. On account of morphological and phenological differences, hybridization between fibreni and macrostigma has been regarded as being, at most, very rare (Gandolfi *et al*., 1991). No genetic data, however, have yet been published to support the differentiation of fibreni and macrostigma in the Posta Fibreno basin.

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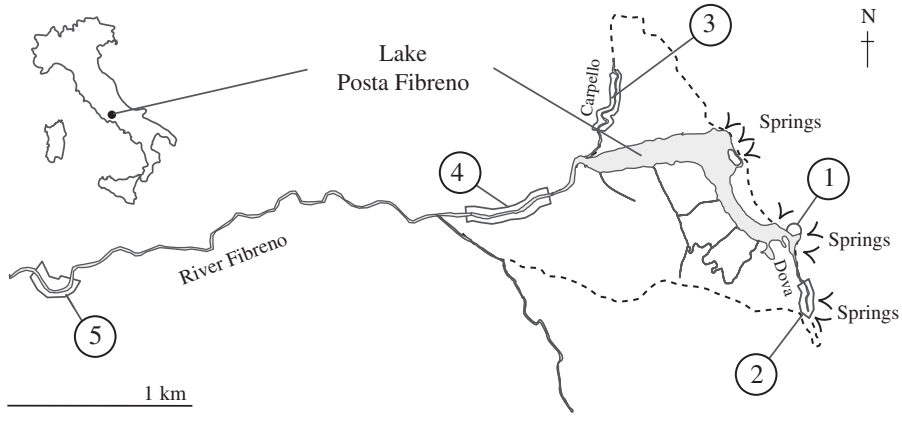

FIG. 2. Map of the Posta Fibreno basin showing the location of the five sites where *Salmo* spp. samples were collected. The boundary of the Riserva Naturale Lago di Posta Fibreno are shown (-----). Main hydrological features (areas with karstic springs and the lake's tributaries Torrente Dova and Torrente Carpello) are also indicated.

Data on allozyme variation (V. Sbordoni, unpubl. data) did not reveal any significant differences between the two forms. Patarnello *et al*. (1994) analysed 593 bp of the mitochondrial genome from a single fibreni and two macrostigma individuals from Posta Fibreno, finding no nucleotide polymorphisms. The only reported genetic difference (although not allelic polymorphism) between fibreni and macrostigma populations is from Alfei *et al*. (1996), who found a significant difference in the size of the nuclear genome (C value) of 11 visually determined fibreni males and nine macrostigma males.

Along with *Salmo carpio* L. 1758 of Lake Garda, *S. fibreni* is one of the two salmonid species strictly endemic to Italy (Gandolfi *et al*., 1991; Kottelat & Freyhof, 2007). Because of its very restricted distribution, it is listed as Vulnerable D2 in the IUCN Red List of Threatened Species (Freyhof & Kottelat, 2008*a*) and as Critically Endangered at a national level (Zerunian, 2002). Moreover, *S. cettii* populations in the Posta Fibreno basin have often been regarded among the most representative of the autochthonous gene pools in the Italian peninsula, and previously were referred to as macrostigma (Gandolfi *et al*., 1991). This taxon is regarded as Near Threatened in the IUCN Red List (Freyhof & Kottelat, 2008*b*) and as Endangered in the Italian red list (Zerunian, 2002). The lake and its surroundings have been designated as a protected area (Riserva Naturale Lago di Posta Fibreno, RNLPF) since 1983, with the main aim of preserving the basin's unique ichthyofauna.

In this study, genetic variation in mitochondrial DNA (mtDNA) sequences and nuclear markers was analysed in 105 individuals sampled across the Posta Fibreno basin in order to: (1) determine the genetic structure of the local *Salmo* spp. forms and (2) assess the degree of reproductive isolation of *S. fibreni* and evaluate its recognition as a distinct endemic species. The analysis of widely applied standard nuclear and mtDNA markers and the inclusion of a hatchery sample in the genetic analyses allowed allochthonous introgression in the Posta Fibreno *Salmo* spp. to be estimated and to take this into account in the examination of the local genetic structure.

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MATERIALS AND METHODS

SAMPLING

Lake Posta Fibreno is a very small karstic lake in central Italy (42° 37′ N; 13° 45′ E). The surface area of the lake is 0·29 km^2 and average depth is 2·5 m (maximum depth: 15 m). The lake is fed by karstic springs with a relatively constant flow (Boni, 2000), and it has clear and well oxygenated water at a constant temperature of *c*. 10° C year-round.

Sampling was carried out from 11 February 2003 to 13 March 2004 (Table I). One hundred and five wild individuals were collected by fish traps (maximum opening diameter = 80 cm, maximum length = 180 cm, mesh size = 15 mm; site 1) or electrofishing (sites 2–5) at five different locations in the Posta Fibreno basin (Table I and Fig. 2). The use of two different sampling techniques, both guaranteeing the full survival of all captured individuals, was justified by the dissimilar hydromorphology of the sites. Site 1 is a small complex of semi-natural underground pools fed by karstic springs. These pools receive water from karstic aquifers and drain into the lake. Site 2 consists of the uppermost reaches (*c*. 50 m) of the lake's tributary Torrente Dova. Sampling sites 3, 4 and 5 are located along streams flowing to or from Lake Posta Fibreno (Fig. 2). All sites are within the RNLPF, except for site 5, which is located just downstream of a small dam (<1 m high), 3 km from the RNLPF boundary (Fig. 2). RNLPF managers consider sites 3 and 4 as well-known spawning sites of the macrostigma population, while fibreni is known to occur in relatively high density and to spawn at sites 1 and 2.

Body mass (M_B) and L_T of sampled fish were immediately measured and samples were photographed. A small fragment (<1 cm^2) of the anal fin was cut, immediately placed in 80% ethanol and then stored at −70° C for DNA extraction. Individuals were sexed by gently stripping the abdomen for ripe gametes. When no release of gametes occurred, the individuals were classified as sub-adults. After sampling, all individuals were released at the site of capture.

An *S. trutta* sample ($n = 23$) from a hatchery stock of predominantly Atlantic origin was used as an external reference to identify introgression of hatchery-origin genes. The sample was obtained on 23 July 2004 from the Centro Ittiogenico della Provincia di Roma, Jenne, Rome, which had been in charge of stocking *S. trutta* in the Posta Fibreno basin until the practice was abandoned in the late 1970s.

ANALYSIS OF PHENOTYPIC TRAITS

According to Zerunian & Gandolfi (1990) and Kottelat & Freyhof (2007), mature individuals of the fibreni and macrostigma forms significantly differ in size (L_T and M_B), number of parr marks (M_P) and number of pigmented spots (S_P), although all of these measurements were reported to show some overlap. In order to objectively assign sampled individuals into phenotypic categories, the above traits were analysed. M_P was defined as the number of large areas (at least larger than the eye) of tenuously pigmented skin crossing the lateral line on one flank of the fish; S_P was defined as the number of areas of the skin with a distinctly darker (blackish or brown-reddish) colour than the background and larger than a single scale on one flank of the fish. Spots on the head of the fish, when present, were not considered. As a certain degree of subjectivity is inevitable in this count, the measurements were taken by two different researchers, and mean values, rounded to the lower integer, were used in further analyses.

For each variable, the normality of distribution was checked within each sampling site by the Shapiro–Wilk (SW) test (correction for multiple tests by the Bonferroni method). Variables with significant SW tests in at least one sample were ln transformed to normalize their distributions. The equality of the means of normally distributed variables across sampling sites was compared by ANOVA test, using the aov function in R [all R functions used in this article belong to the basic {stats} package, R Development Core Team; http://www.R-project.org/]. ANOVA assumptions about normal distribution and homoskedasticity of residuals were visually checked by inspecting plots of observed *v*. theoretical quantiles (*Q*–*Q* plot) and residuals *v*. fitted values. *Post hoc* pair-wise comparisons were performed by Tukey's HSD (R function:

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TABLE I. Samples and external morphology. Morphological traits have been measured on adult *Salmo* spp. only. Adult individuals with available data for all four morphological traits were included in multivariate analyses (principal component analysis and cluster analysis)

Sampling site	1	2	3	4	5
Date (2004)	8 March	13 March	11 February	19 February	27 February
n	15	16	46	7	21
Sub-adults	0	1	0	1	1
Males	6	9	30	3	6
Females	9	6	5	3	13
Sex not determined	0	0	11	0	1
Pigmented spots (S_P)					
n	14	15	31	5	18
Mean	14·2	11·3	77·5	41·6	54·7
S.D.	8·3	6·8	50·8	20·1	28·8
Minimum	4	0	23	22	19
Maximum	32	27	270	70	124
Parr marks (M_P)					
n	13	15	23	4	16
Mean	7·3	8·0	11·2	13·2	10·4
S.D.	0·6	0·9	1·2	2·2	1·6
Minimum	6	6	8	11	7
Maximum	8	9	13	16	13
Total length (L_T, cm)					
n	15	15	35	6	9
Mean	15·2	16·0	29·6	26·7	25·0
S.D.	3·1	3·2	5·7	5·5	4·4
Minimum	11	12	17	21	16
Maximum	23	25	42	36	35
Body mass (M_B, g)					
n	15	15	35	6	9
Mean	39·4	49·2	313·3	243·7	184·3
S.D.	23·8	33·6	177·6	182·4	91·7
Minimum	15	18	55	108	44
Maximum	112	153	850	580	430
Multivariate morphological analyses					
n	12	15	23	3	16
Fibreni	12	14	0	0	1
Macrostigma	0	1	23	3	15

TukeyHSD). When the SW test remained significant after ln transformation in at least one sample, Kruskal–Wallis test (R function: kruskal.test) was used to compare sample medians and *post hoc* pair-wise comparisons were performed by Mann–Whitney *U*-tests (R function: wilcox.test). In the latter case, correction for multiple tests was performed by the Bonferroni method.

In order to obtain a synthetic description of external morphology, a principal component analysis (PCA) was performed on the four phenotypic variables, L_T, M_B, M_P and S_P. Prior to the PCA calculations, variables were ln transformed when it allowed normalization of their distribution and were scaled by dividing each variable by its S.D. PCA was performed using the prcomp function in R. Only sexually mature individuals whose photographs allowed confident

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determination of both S_P and M_P were included in the morphological analyses (Table I). An operational classification of individual phenotypes into discrete categories was obtained by a *k*-means cluster analysis (R function: kmeans; default method, 1000 random starts) of the complete PCA scores (PC1–PC4).

GENOTYPING

DNA was extracted from the fin tissue by overnight incubation in C-Tab buffer (Doyle & Doyle, 1987) containing 3 U ml^{-1} K-proteinase and by chloroform extraction. The entire mtDNA control region (CR) was PCR-amplified using *PST* and *FST* primers (Cortey & García Marín, 2002) and sequenced in both directions, using the same primers employed for amplification, on a sub-set of 48 individuals: 12 specimens were analysed from site 1, 11 from site 2, 16 from site 3, five from site 4 and four from site 5.

All sampled fish (105 from Posta Fibreno and 23 *S. trutta* from the hatchery) were genotyped at 12 microsatellite loci: BS131, 543AE, T3-13 (Estoup *et al*., 1998), Str15 (Estoup *et al*., 1993), MST591, MST79, MST28 (Presa & Guyomard, 1996), Strutta12, Strutta58 (Poteaux *et al*., 1999), Ssa410, Ssa408 (Cairney *et al*., 2000) and SsaD71 (King *et al*., 2005). PCR conditions followed the cited literature. One primer of each pair was end-labelled with fluorescent dyes FAM (Invitrogen; www.invitrogen.com), NED or VIC (Applied Biosystems; www. appliedbiosystems.com). Fragment analysis was performed on ABI Prism® 3100 Genetic Analyzer (Applied Biosystems).

Retinoic lactate dehydrogenase (ldh-c1) is a highly polymorphic enzyme in the *S. trutta* complex. The two commonest alleles are **100*, found at very high frequency in Mediterranean populations, and **90*, which is naturally restricted to north-west Europe (Hamilton *et al*., 1989). Most hatchery stocks in Europe show a high frequency of the allele **90*. The frequency of allele **90* is therefore considered a good proxy for the introgression of hatchery genes in natural Mediterranean populations (Machordom *et al*., 1999; Berrebi *et al*., 2000; McMeel *et al*., 2001; Nonnis Marzano *et al*., 2003). Assay of the enzyme-coding gene *ldh-c1** by PCR restriction fragment length polymorphism (RFLP) allowed an estimate of allochthonous introgression directly comparable with allozyme-based literature to be obtained. Genotyping followed the protocol by McMeel *et al*. (2001), consisting of the PCR amplification of a 440 bp fragment, followed by digestion with *bsl*I bacterial endonuclease (New England Biolabs; www.neb.com).

ANALYSIS OF MTDNA CONTROL REGION DATA

Nucleotide sequences of the mtDNA CR were aligned and edited in MEGA 5.0 (Tamura *et al*., 2011). The basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/) was used to compare the obtained sequences to the ones published in the NCBI GenBank and to produce a neighbour-joining (NJ) tree on the matrix of Jukes–Cantor nucleotide distances.

ANALYSIS OF POLYMORPHISM AT NUCLEAR LOCI

The software package Genetix 4.05.2 (Belkhir *et al*., 1996–2001) was used to calculate genetic variation parameters (observed heterozygosity and unbiased expected heterozygosity), allelic frequencies, *F*-statistics (significance tested by 1000 permutations) and to test for genotypic disequilibrium between pairs of loci (1000 permutations). Correction for multiple tests was performed by the Bonferroni method.

GENETIC STRUCTURE

A factorial correspondence analysis (FCA) was performed in Genetix 4.05.2 in order to explore the distribution of allelic diversity at nuclear loci (microsatellites and *ldh-c1**).

Bayesian assignment tests implemented in the software Structure 2.3.3 (Pritchard *et al*., 2000), were used to estimate the number of genetic clusters and to evaluate the degree of admixture among them. Structure is based on a Bayesian approach and uses a Monte-Carlo

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Markov-Chain (MCMC) algorithm to assign individuals to K genetic clusters. The admixture model (with both correlated and uncorrelated allelic frequencies among populations) implemented in Structure was used to obtain estimates (and 90% credibility intervals) for the proportion of ancestry (Q) of each individual genotype in each of the K clusters. MCMCs were run for 1 000 000 generations after 100 000 generations of burn-in. In order to estimate the number of genetically distinct clusters (K), analyses were run for several values of K. Ten replications were performed for each value of K. The value of K corresponding to the highest mean probability of the data was chosen as the best estimate for the number of genetic clusters (after checking for consistence across replicates). The value of the statistic ΔK was used to identify the greatest rate of change between each subsequent K (Evanno *et al*., 2005) and thus determine the uppermost level of the structure.

The results from the Structure analysis were used to test the hypothesis that the degree of genetic admixture was equal across sampling sites by performing a Kruskal–Wallis test on the highest Q value for each individual. *Post hoc* pair-wise comparisons were performed by Mann–Whitney U-tests with Bonferroni correction.

TEST OF GENETIC INTROGRESSION *V*. HYBRIDIZATION

The hypothesis that reproductive isolation between the fibreni and macrostigma forms does not break down beyond the first hybrid generation (F1) was explicitly tested by the algorithm implemented in the software NewHybrids (Anderson & Thompson, 2002). This programme implements a Gibbs sampler to estimate the posterior probability (P) that genetically sampled individuals fall into each of a set of user-defined hybrid categories, namely: parental from gene pools 0 or 1 (P0 or P1), P0 × P1 (F1), F1 × F1 (F2), F1 × P0 or F1 × P1 backcrosses (BX0 or BX1). Computations were performed assuming Jeffreys-like priors for allele frequencies (h) and mixing proportions (p) and without extra priors on specific allele frequency information. Three independent simulations were run to check for consistency in the convergence of Markov chains, with a burn-in of 100 000 steps, followed by a sampling period of 1 000 000 iterations. The rationale of the test was as follows: if F1 hybrids between the two forms were not fertile, all individuals should belong to one of the two parental categories (P0 and P1) or to the F1 category. The presence of individuals whose sum of posterior probabilities for P0, P1 and F1 was $<0{\cdot}05$ was therefore taken as evidence that genetic introgression proceeds further than just occasional F1 crosses.

RESULTS

EXTERNAL MORPHOLOGY

Morphological analyses were conducted on 90 mature fish; sex was not determined for 12 individuals and only three individuals were classified as sub-adults (Table I). As sexual maturity was determined by the presence of ripe gametes, it is possible that a few adult specimens were actually sampled after the spawning season was over and therefore incorrectly assigned to sub-adult class. The limited number of sub-adults observed ($n = 3$), however, made this potential problem irrelevant.

SW tests indicated non-normality of distribution ($P < 0{\cdot}05$) for all variables in at least one sampling site. The distribution of ln L_T, ln M_B and ln S_P was consistent with a normal distribution in all samples (SW, all $P > 0{\cdot}05$). Both M_P and ln M_P were non-normally distributed in three samples (SW, $P < 0{\cdot}05$ in sampling site 1 after Bonferroni correction). Three variables (M_P, ln L_T and ln M_B) showed a bimodal global distribution when data were pooled across sampling sites, although for none of them a distribution gap was apparent [Fig. 3(a)–(c)]. The size of sampled fish ranged from $L_T = 9{\cdot}5$ cm, $M_B = 8$ g to $L_T = 42$ cm, $M_B = 850$ g. The

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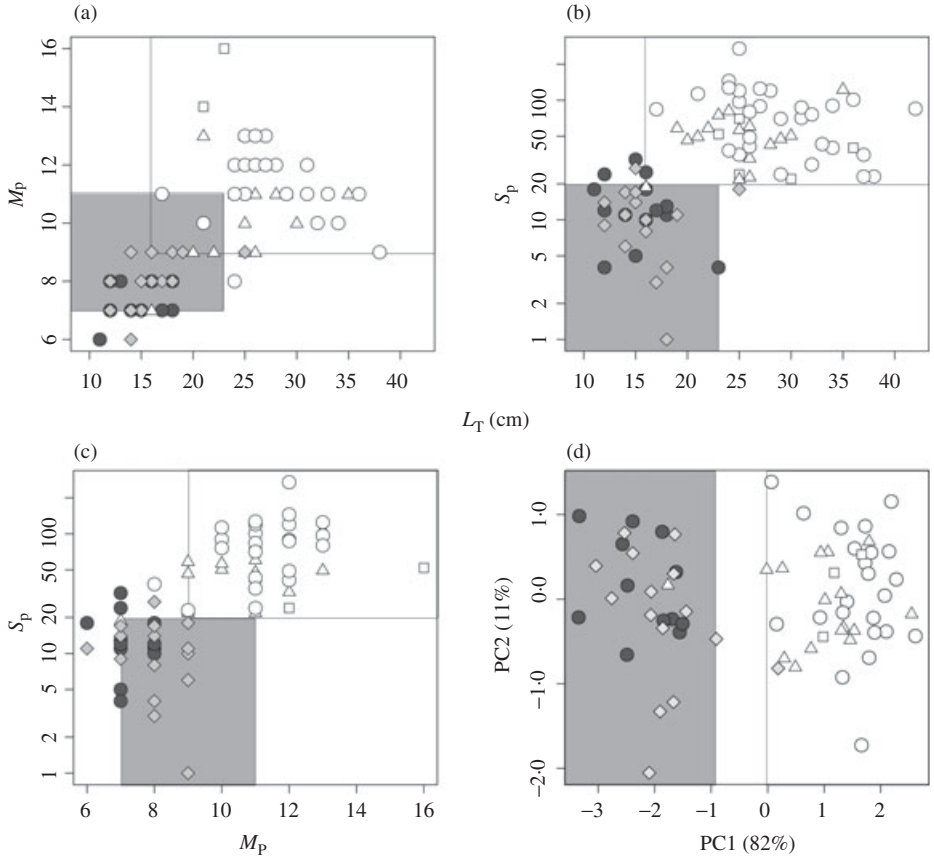

FIG. 3. External morphology. (a, b, c) Pair-wise scatterplots of total length (L_T), number of parr marks (M_P) and number of pigmented spots (S_P). (d) Scatterplot of principal components 1 (PC1) and 2 (PC2) from the principal component analysis performed on four phenotypic traits. Samples from each sampling site are indicated (●, 1; ◇, 2; ○, 3; □, 4; △, 5;). Ranges of the macrostigma (□) and fibreni (▨) forms in the phenotypic space [(a, b, c) according to Zerunian & Gandolfi (1990) and Kottelat & Freyhof (2007) and (d) according to the operational definition based on *k*-means cluster analysis].

smallest mature individual was a male caught at site 1 ($L_T = 11$ cm, $M_B = 15$ g; Table I).

Inspections of Q–Q plots and fitted v. residuals plots did not reveal any obvious deviation from ANOVA assumptions. The mean size of individuals strongly differed across sampling sites ($\ln L_T$: $F = 47{\cdot}5$, $P < 0{\cdot}001$; $\ln M_B$: $F = 52{\cdot}1$, $P < 0{\cdot}001$). The mean size of sexually mature fish sampled at sites 1 and 2 was significantly smaller than at sites 3, 4 and 5 (Tukey's HSD: all $P < 0{\cdot}001$ for both $\ln L_T$ and $\ln M_B$). Very small mature individuals ($M_B < 30$ g, $L_T < 15$ cm) were only sampled at sites 1 and 2 and only three adults with $M_B < 90$ g were caught at sites 3, 4 and 5. On the other hand, no fish weighing >170 g were sampled at sites 1 and 2. Also, fish from site 5 were significantly smaller than fish from site 3 (Tukey's HSD: $P < 0{\cdot}01$ for both $\ln L_T$ and $\ln M_B$).

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A similar difference was also found in colour pattern traits, as samples from sites 1 and 2 showed significantly lower mean values for both S_P and M_P (all *post hoc* tests: $P < 0{\cdot}05$) (Table I). No significant differences in M_P and S_P were observed between sites 1 and 2 or among sites 3, 4 and 5.

The combined distributions of size (L_T) and colour pattern traits, superimposed with the ranges of variation reported for macrostigma and fibreni by Zerunian & Gandolfi (1990), Gibertini *et al*. (1990) and Kottelat & Freyhof (2007) are illustrated in Fig. 3(a)–(c). Values outside the reported ranges were observed for both S_P and M_P in sites 1 and 2, and a few individuals did not fall into any of the two described ranges in bivariate spaces [*e.g*. four individual with $S_P > 20$ and $M_P < 9$ were observed; Fig. 3(c)]. Most individuals from sites 1 and 2, however, fell within the ranges of variation reported for the fibreni form, while the macrostigma traits, as described from literature, are most common at sites 3, 4 and 5. Furthermore, the plot of L_T and S_P values [Fig. 3(b)] showed two non-overlapping clouds, widely superimposed to the ranges attributed to the fibreni and macrostigma forms in earlier studies.

All morphological variables were positively correlated with each other. Besides the obvious, near-perfect, correlation between L_T and M_B (Pearson's coefficient $= 0{\cdot}96$, $P < 0{\cdot}001$), both colour pattern variables (ln S_P and M_P) showed a positive correlation with body size when data from all individuals were considered altogether [M_P *v*. L_T: Pearson's coefficient $= 0{\cdot}69$, $P < 0{\cdot}001$; S_P *v*. L_T: Pearson's coefficient $= 0{\cdot}58$, $P < 0{\cdot}001$; Fig. 3(a), (b)].

PCA allowed deriving a synthetic descriptor of the phenotypic variation, as the first principal component (PC1) explained 82% of the total variance. Moreover, two phenotypic groups could be clearly distinguished in the PCA space [Fig. 3(d)], with only a single individual from site 2 placed in a somewhat intermediate position on PC1. In the *k*-means analysis ($k = 2$), the two groups consisted of all phenotypes with PC1 $< -0{\cdot}5$ and all phenotypes with PC1 $> 0{\cdot}1$, respectively. The between-group sum of squares was 71% of the total sum of squares. The loadings of the four phenotypic variables into PC1 are all positive (ln M_B: 0·53; ln L_T: 0·52; M_P: 0·48; ln S_P: 0·46), all the variables being strongly and significantly correlated with PC1 (ln M_B: Pearson's coefficient $= 0{\cdot}95$; ln L_T: Pearson's coefficient $= 0{\cdot}94$; M_P: Pearson's coefficient $= 0{\cdot}87$; ln S_P: Pearson's coefficient $= 0{\cdot}83$; all $P < 0{\cdot}001$). Therefore, low values of PC1 are associated with small sexually mature individuals with fewer parr marks and spots, thus corresponding very well to the available descriptions of the fibreni form and, *vice versa*, higher values of PC1 match well with the macrostigma form. In the following, the two clusters thus obtained are used as operational definitions of the macrostigma and fibreni phenotypes. The number of individuals assigned to each of the groups at each sampling site is given in Table I.

mtDNA POLYMORPHISM

A fragment of 999 bp of the mtDNA CR was successfully sequenced in 48 wild-caught individuals from the Posta Fibreno basin. All the individuals shared the same haplotype (GenBank accession number JQ314219). The most similar sequence in the GenBank database (last searched 30 October 2011) was ADcs20 (GenBank accession number AY836349.1), a haplotype first described by Cortey *et al*. (2004) in Mediterranean *S. trutta* differing by two A-G transitions. The NJ tree showed that the novel haplotype is clearly included in the AD mitochondrial haplogroup (Bernatchez, 2001).

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*LDH-C1** POLYMORPHISM

The global frequency of the Atlantic allele *ldh-c1**90 was 0·09 in natural samples, ranging from 0·00 in the very small sample from site 4 to 0·21 in site 5, outside the RNLPF. Within the RNLPF, the highest frequency of the allochthonous allele was found at site 2 (0·12).

MICROSATELLITE POLYMORPHISM

Of the 12 microsatellite loci analysed, 11 were polymorphic in the samples from Posta Fibreno. Locus *MST28* was monomorphic, as all samples (including the hatchery samples) shared the same allele. The mean observed heterozygosity across loci in the natural sample was 0·543, ranging from 0·477 in site 1 to 0·607 in site 2. Values of F_{IS} were non-significantly positive for all samples ($P > 0{\cdot}05$) except for sample from site 2, which showed a non-significant heterozygosity excess ($F_{IS} = -0{\cdot}052$, $P > 0{\cdot}05$). No evidence of linkage disequilibrium was found in the natural samples, as all the possible combinations within samples were non-significant after correction for multiple tests.

GENETIC STRUCTURE

The genetic structure of the Posta Fibreno samples was analysed by including a reference *S. trutta* sample from a hatchery strain used for stocking in the same region. The FCA on all 13 nuclear loci (Fig. 4) separates the Posta Fibreno samples from hatchery samples on the first factor. A few individuals, mostly from sample 5, appear to be intermediate between the Posta Fibreno main cluster and the hatchery cluster. The Posta Fibreno samples were spread along factor 2: all individuals with macrostigma phenotype were assigned high scores, while all samples from site 1 (all fibreni phenotype) and the individual from site 5 with fibreni phenotype were assigned lower scores. Thus, there was an apparent association between phenotypic and genetic clustering for all samples from sites 1, 3, 4 and 5. Individuals from sample 2 (one macrostigma and 14 fibreni phenotypes), however, were scattered along factor 2.

The individual clustering pattern and admixture proportions obtained following the uncorrelated frequency model and the correlated frequency model in Structure were virtually the same. Results from the uncorrelated frequency model are reported here. When Posta Fibreno and hatchery samples were analysed together in Structure, testing values of K from 1 to 10, the model with three genetic clusters ($K = 3$) corresponded to the highest probability of the data [Fig. 5(a)], while the model with $K = 2$ returned the highest ΔK [Fig. 5(b)]. In the $K = 2$ model, one cluster corresponds to the hatchery sample, with a few admixed individuals in the Posta Fibreno samples [Fig. 5(c)]. In the $K = 3$ model [Fig. 5(d)], the hatchery cluster remained virtually unchanged, whereas the Posta Fibreno cluster was split in two: all but one individual with macrostigma morphotype were assigned to one of the new clusters with $Q > 0{\cdot}90$ and all but one individual with fibreni phenotype from site 1 and the one from site 5 were assigned to the second new cluster with $Q > 0{\cdot}90$. One individual from site 1 (fibreni phenotype) and one from site 5 (macrostigma phenotype) were assigned intermediate Q values (not assigned to any of the clusters with $Q > 0{\cdot}90$). Consistent with the results from FCA, samples from site 2

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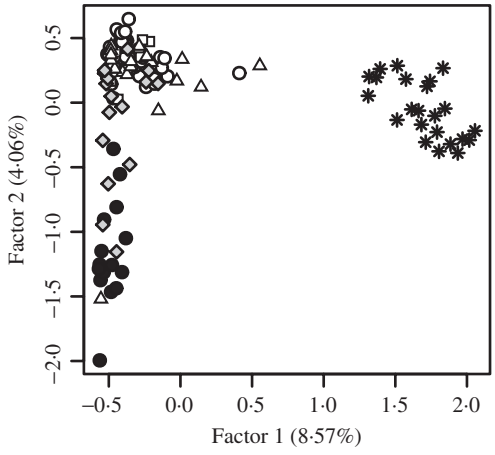

FIG. 4. Scatterplot of factor 1 and factor 2 of a factorial correspondence analysis based on 12 polymorphic nuclear loci (11 polymorphic microsatellite loci and *ldh-c1*) from *Salmo* spp. The number in parentheses indicates the percentage of inertia contained in each factor. Samples from each sampling site are indicated (●, 1; ◇, 2; ○, 3; □, 4; △, 5; ✱, hatchery).

were assigned to both clusters, with five individuals (31%) assigned intermediate values.

In order to further investigate the genetic structure within the Posta Fibreno samples, a second analysis was performed in Structure after removing all individuals that were assigned a $Q > 0{\cdot}05$ for the allochthonous cluster in the first analysis with $K = 2$. In this second Structure analysis, which tested K values from 1 to 5, $K = 2$ corresponded to both the highest probability of the data and the highest ΔK [Fig. 6(a), (b)]. The assignment of individuals to the two Posta Fibreno clusters in this last analysis [Fig. 6(c)] was virtually the same as in the first analysis with $K = 3$ (Pearson's correlation coefficient = 1·00).

In the latter analysis, the mean of the highest Q value in samples 1, 3, 4 and 5 ranged from 0·98 to 0·99, while in sample 2 it was 0·89. The Kruskal–Wallis test rejected the equality of admixture across sites with $P < 0{\cdot}001$. Pair-wise Mann–Whitney U-tests were significant for comparisons of site 2 with sites 1, 3 and 5 (sample 4 has a very small sample size, $n = 7$). Results from the latter analysis were used to compare the genetic and morphological assignment by plotting Q values for the macrostigma cluster against the scores of the PC1 calculated on phenotypic traits [Fig. 6(d)]. While the global correlation was obvious (Pearson's coefficient = 0·744, $P < 0{\cdot}001$), no apparent correlation was found for those samples with intermediate ($Q < 0{\cdot}95$ in both clusters) Q values (Pearson's coefficient = 0·29, $P > 0{\cdot}05$).

GENETIC INTROGRESSION *V*. HYBRIDIZATION

The NewHybrids software was run on the same set of samples retained for the second Structure run (individuals with significant allochthonous genetic profiles were removed). The three independent runs gave virtually identical results and mean posterior probabilities (P) from the three runs are reported. Most individuals (62%) were assigned to one of the two parental categories with $P > 0{\cdot}90$ (53% with $P > 0{\cdot}95$).

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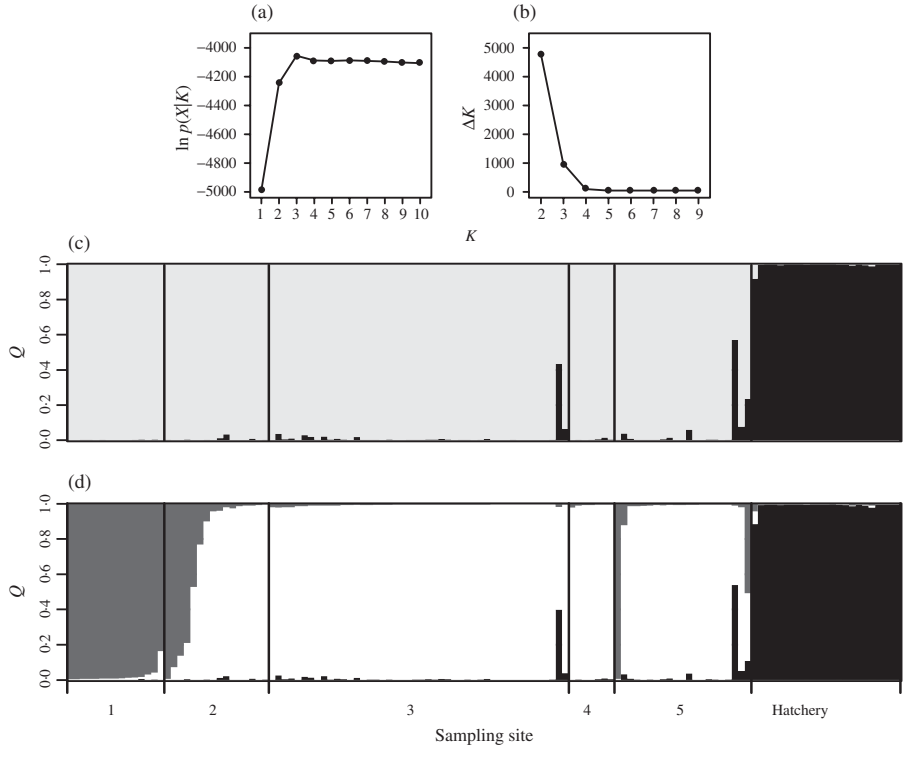

FIG. 5. Results from Structure 2.3.3 analysis including all Posta Fibreno samples and the hatchery reference sample of *Salmo* spp. (a) Estimated ln of $p(X|K)$ (probability of the data given the number of clusters) as a function of K (number of clusters). (b) ΔK statistic (Evanno *et al*., 2005) as a function of K. (c) Assignment of individual genotypes with $K = 2$. (d) Assignment of individual genotypes with $K = 3$. In (c, d), each vertical bar represents an individual genotype and the height of shaded sub-bars represents the proportion of ancestry (Q) for the individual genotype in each cluster.

Only two, however, were sampled at site 2 (one was assigned to fibreni and one to macrostigma parental categories). For none of the sampled individuals, the hybrid category F1 was the most probable (*i.e*. the category with the highest P). For 15 individuals, the most probable category was the backcross F1 × macrostigma (P ranging from 0·49 to 0·92), while for six individuals the most probable category was F1 × fibreni (P ranging from 0·49 to 0·79). Six individuals (five from site 2 and one from site 5) had summed posterior probabilities for parental categories (P0 and P1) and F1 below the 0·05 threshold. The latter result was taken as evidence that introgression between the macrostigma and fibreni gene pools progresses beyond F1.

DISCUSSION

AUTOCHTHONY OF THE POSTA FIBRENO GENE POOLS

Traces of the genetic introgression from commercial allochthonous gene pools are evident in the Posta Fibreno basin, as the allele *ldh-c1**90 (typically fixed in the

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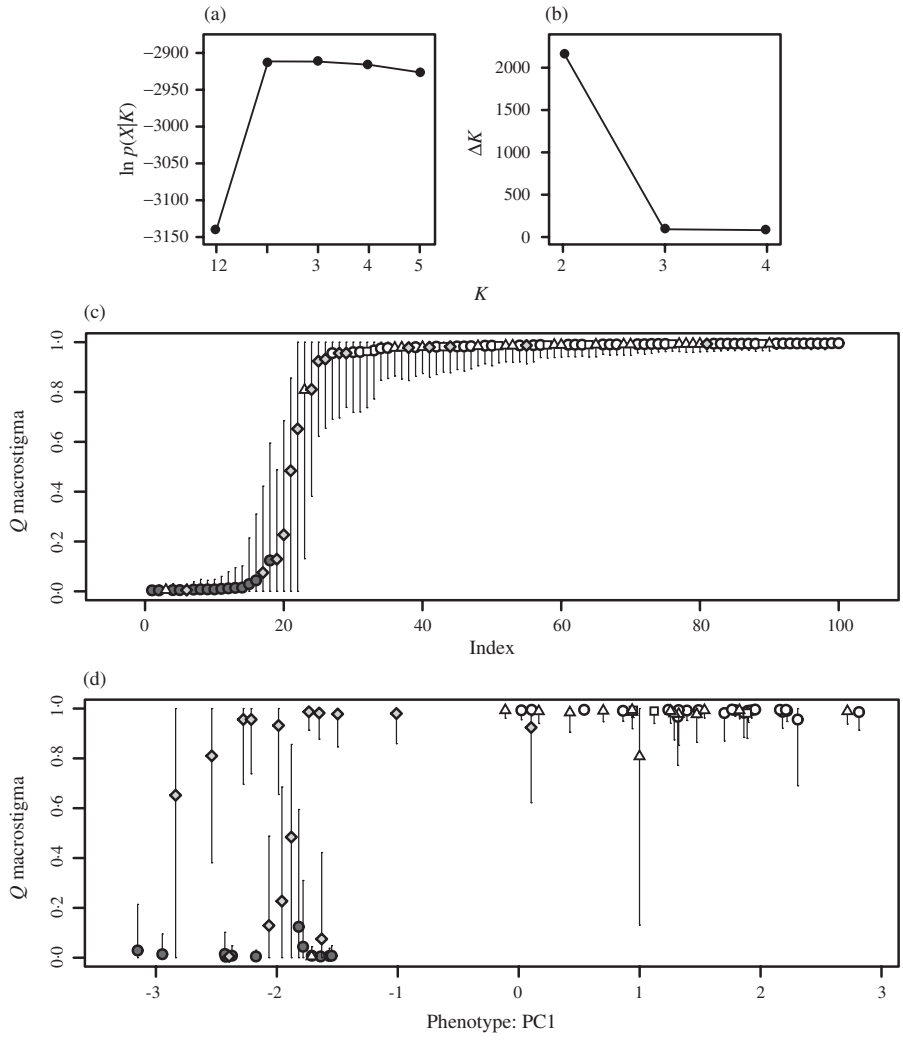

FIG. 6. Results from Structure 2.3.3 analysis including only Posta Fibreno samples of *Salmo* spp. with no significant hatchery ancestry. (a) Estimated ln of $p(X|K)$ (probability of the data given the number of clusters) as a function of K (number of clusters). (b) ΔK statistic (Evanno *et al*., 2005) as a function of K. (c) Plot of the estimated ancestry (Q) in the macrostigma cluster for each individual genotype ($K = 2$); genotypes were sorted from the lowest to the highest Q. (d) Scatterplot of the estimated ancestry (Q) in the macrostigma cluster for each individual genotype ($K = 2$) against the corresponding score for individual phenotype in first principal component (PC1) from the principal component analysis based on phenotypic traits. (c, d) Error bars include the estimates 90% credibility interval for Q (sampling sites: ●, 1; ◇, 2; ○, 3; □, 4; △, 5).

strains from commercial hatcheries) was observed in the fish from most of the sampling sites and a few multilocus nuclear genotypes were genetically similar to those of the fish from the hatchery. All of the 48 mitochondrial sequences analysed belong to a haplotype which has never been observed elsewhere, and is clearly related to the autochthonous Mediterranean AD haplogroup. Several central Italian *S. trutta* populations have been assayed for mitochondrial markers and *ldh-c1** polymorphism

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by Nonnis Marzano *et al*. (2003) and Caputo *et al*. (2004). These authors found frequencies of the *ldh-c1* *90 higher than in the sample from Posta Fibreno at 31 of the 32 sampled sites in central Italy (*ldh-c1* *90 was not found in a single sample with $n = 10$). Moreover, clearly allochthonous mtDNA was found in most sites sampled by Nonnis Marzano *et al*. (2003) and Caputo *et al*. (2004). Similarly, Berrebi *et al*. (2000) reported frequencies of *ldh-c1* *90 > 0·10 in 10 of their 12 samples from south-western France. Complete absence of the allochthonous *ldh-c1* *90 allele was observed in two small population samples ($n = 15$ and $n = 6$) of *Salmo trutta fario* (*Salmo cenerinus* Chiereghin 1847; Kottelat & Freyhof, 2007) from north-western Italy: in a sample of *S. carpio* from Lake Garda and in several population samples of *Salmo marmoratus* Cuvier 1829 from north-eastern Italy (Giuffra *et al*., 1996). On the other hand, Meraner *et al*. (2007) found significant or strong mtDNA introgression in *S. marmoratus* from South Tyrol. Similarly, Pujolar *et al*. (2011) reported allochthonous mtDNA in phenotypically selected samples of *S. marmoratus* from northern Italy. The last study employed microsatellite data to compare their wild-caught samples to a hatchery reference, as in this study. Their results can be compared directly, therefore, to the present results. Pujolar *et al*. (2011) reported that the proportion of individuals with an estimated fraction of hatchery gene pool >0·10 ranged from 3·3 to 33·0% in their samples, while this proportion is 2·8% in the present overall sample, and only 1·2% within the RNLPF.

One of the lowest levels of introgression from stocked commercial strains in the central Mediterranean basin is therefore observed in the Posta Fibreno populations. This result shows that the cessation of stocking in the Fibreno basin established *c*. 30 years ago has been effective in preserving the local gene pools.

GENETIC STRUCTURE AMONG AUTOCHTHONOUS POPULATIONS

The genetic analyses showed that the autochthonous *Salmo* spp. populations of the Posta Fibreno basin consist of two distinct gene pools [Figs 4, 5(d) and 6(d)]. The morphologic analysis has also shown that a very limited set of phenotypic traits (namely the body size and the number of parr marks and pigmented spots along the flanks) can be used to objectively sort sexually mature individuals in two distinct phenotypic classes, with very little ambiguity [Fig. 3(d)]. These results are in good agreement with the descriptions provided by Zerunian & Gandolfi (1986, 1990) for the fibreni (*S. fibreni*) and macrostigma (*S. cettii*; Kottelat & Freyhof, 2007) forms, although some minor discrepancies were observed [Fig. 3(a)–(c)]. Phenotypic and genotypic classes are found in nearly-perfect association at four of the five sampling sites [Fig. 6(d)]. The analyses therefore show that the fibreni form, strictly endemic to Lake Posta Fibreno, and macrostigma form, referable to the Tyrrhenian *S. cettii* of the Italian peninsula, correspond to two distinct autochthonous gene pools.

Nonetheless, genetically intermediate individuals were observed, in particular, at sampling site 2. Data analyses indicated that genetic introgression proceeds beyond the F1 level, suggesting that reproductive isolation is clearly not complete. Hybridization and introgression between distinct taxa, even phylogenetically distant species, are very common in salmonids (Garcia de Leaniz & Verspoor, 1989; Matthews *et al*., 2000; Bettles *et al*., 2005; Kahilainen *et al*., 2011). Among salmonids of the Italian

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fauna, *S. marmoratus* is usually regarded as a true species, although it has extensively introgressed with allochthonous *S. trutta* strains in north-eastern Italy in the last century (Meraner *et al.*, 2010). Therefore, the lack of reproductive isolation cannot be considered to argue against the recognition of *S. fibreni* as a distinct species, endemic to the Posta Fibreno basin. In fact, the macrostigma and fibreni forms have occurred in sympatry for centuries (Salviani, 1554; Carbone, 1965) and the present data show that this has not caused any complete merging of the two gene pools. Ecological specialization (*e.g.* temporal and spatial discrepancies in spawning) might have played an important role in limiting gene flow between the two forms, favouring the maintenance of genetic differentiation despite the lack of complete reproductive isolation.

INFLUENCE OF HABITAT FEATURES ON HYBRIDIZATION

The higher degree of admixture observed at site 2 is consistent with its environmental features, which are intermediate between site 1 on one side and sites 3, 4, and 5 on the other side. Site 1 consists of underground karstic pools, the preferred feeding and spawning habitat for *S. fibreni*, while sites 3, 4 and 5 are streams, the habitat where macrostigma trout are expected to spawn (Fig. 2). Consistently, most individuals at site 1 are morphologically and genetically assigned to the fibreni clusters [Fig. 6(d)], as well as the vast majority of samples from sites 3, 4 and 5 are assigned to the macrostigma clusters. On the other hand, site 2 is found close to the karstic springs, feeding a very short stream (Fig. 2). It is therefore the only sampling site where spawning of both the macrostigma and fibreni forms can be expected to occur.

Influence of habitat features on the degree of admixture of populations has been observed in areas of hybridization among salmonid species (Charles *et al.*, 2005; Rasmussen *et al.*, 2010). As information about other ecological differences between macrostigma and fibreni (*e.g.* feeding ecology) is lacking, the role played by the selection of spawning sites in maintaining the genetic differentiation between the two forms can only be hypothesized. Under this hypothesis, frequent interbreeding might only occur at sites where suitable conditions for the spawning of both phenotypes are found (*e.g.* stream stretches very close to karstic springs, as at site 2). An interesting hypothesis is that, as fibreni males are much smaller than macrostigma, they can be expected to suffer a competitive disadvantage at spawning sites, so that hybridization could be asymmetric. Unfortunately, as variation at mtDNA was not found, this hypothesis cannot be tested with the present data.

MECHANISMS MAINTAINING GENETIC DIFFERENTIATION

Selection for spawning sites alone would not guarantee the long-term maintenance of genetic differentiation. In fact, intensive migration among spawning sites (*e.g.* between sites 1 and 2) would eventually bring to homogenization of allelic frequencies. On the other hand, if philopatry of fibreni was very strong, spawning individuals at site 2 should be expected to represent a unique gene pool, albeit possibly originated by hybridization. No evidence for this, however, was found in the genetic analyses. Therefore, natural selection may also be suggested to be acting to maintain the individuality of the fibreni gene pool. In a plausible scenario, if hatching

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success at typical fibreni spawning sites (*e.g.* site 1) was lower for admixed genotypes than for the parental genotypes, this would preserve the genetic distinctiveness of source populations. Fibreni females lay very few eggs that are larger than in other *S. trutta* forms (Gibertini *et al.*, 1990), a condition typically occurring in the cave-adapted fish (Poulson, 2001). This suggests that the fibreni form may be especially adapted to develop in the unusual, buffered conditions found at deep karstic springs.

ASSOCIATION OF PHENOTYPE AND GENOTYPE IN ADMIXED INDIVIDUALS

While the association between genetic and phenotypic assignments was very strong for most individuals, such a relationship was not apparent for those individuals with admixed genotypes [Fig. 6(d)]. This suggests that the markers may not be dense enough to track the phenotypic variation, and that the phenotypic traits which were scored depend on a few genetic loci. It cannot be ruled out, however, that phenotypic plasticity determines, at least partially, the development of the fibreni and macrostigma phenotypes (described in terms of size and colour pattern). The influence of environmental cues on the fish size at sexual maturity in salmonids is well known (Jonsson & Jonsson, 2011) and both the colour pattern variables that were measured correlate with body size. Moreover, colour pattern may itself be influenced by environmental cues (Bourke *et al.*, 1997). Under this hypothesis, the genetic and phenotypic differentiation found in the Posta Fibreno *Salmo* spp. forms would be only indirectly related, for example, through behavioural selection of spawning sites, which would, in turn, influence external morphology *via* phenotypic plasticity.

In more detail, most admixed individuals found at site 2 had a distinctively fibreni phenotype, while only one can be considered to be intermediate, and one was assigned to the macrostigma phenotype [Fig. 6(d)]. Phenotypic plasticity could be an explanation for this finding, implying that the scored phenotype would be more strictly related to the site where an individual was caught than to its genotype. For want of more detailed information about the biology of these populations, however, at least another tentative hypothesis can be drawn. Considering that (1) fish from site 2 were sampled on 13 March, and all but one fish collected brought mature gametes and (2) the typical spawning season of macrostigma ends in February, while fibreni females are reported to lay eggs through, at least, April, the finding that most individuals sampled at site 2 were phenotypically fibreni, though showing admixed genotypes, might be explained by assuming that site 2 is used mostly as a spawning site, and that the timing of reproduction is strictly associated with phenotypic features (*e.g.* because size at maturity and synchronization of gamete maturation may be developmentally correlated).

In conclusion, the data confirm that *Salmo* spp. populations of the Posta Fibreno basin have a high degree of autochthony, and show that the two described forms, fibreni and macrostigma, correspond to two well defined gene pools. The results, therefore, indicate that the recognition of *S. fibreni* as a distinct, endemic species is biologically meaningful and managerially useful. Consistent to the current taxonomy (Kottelat & Freyhof, 2007), the macrostigma form from Posta Fibreno should instead be regarded as a population of *S. cettii*.

Genetic introgression between the two gene pools, however, was observed, whose frequency seem to be correlated with the environmental features of the spawning

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sites. Although several aspects of the biology of Posta Fibreno *Salmo* spp. forms still awaits further investigation, the interplay of selection for spawning sites, philopatry and natural selection may be argued to be acting to maintain genetic differentiation despite the lack of reproductive isolation. Therefore, the Posta Fibreno basin, besides representing a refuge of autochthonous biodiversity, is also a valuable micro-scale model for adaptive divergence and ecologically mediated hybridization in salmonids. Future conservation measures taken in the Posta Fibreno basin should fully account for the crucial importance of habitat variation for the maintenance of biological diversity.

This study was supported by research grants from the Direzione Regionale Ambiente, Regione Lazio and the Riserva Naturale Lago di Posta Fibreno (RNLPF) to V.S. Also, P.G. and A.G. were partially funded by the Autonomous Province of Trento (Italy) within the ACE-SAP project (regulation number 23, 12 June 2008, of the University and Scientific Research Service). The authors are grateful to A. Lecce and other staff members at RNLPF for participating in sampling. Comments from two anonymous reviewers on the first version of the manuscript significantly contributed to improve the quality of this article. Finally, the authors thank D. B. Neale for the linguistic revision of the manuscript.

References

Alfei, L., Cavallo, D., Eleuteri, P., Colombari, P. T., Ferri, A., Onali, A. & De Vita, R. (1996). Nuclear DNA content assessment in *Salmo fibreni* living in the Italian lake of Posta Fibreno. *Journal of Fish Biology* **48,** 1051–1058. doi: 10.1111/j.1095-8649.1996.tb01803.x

Anderson, E. C. & Thompson, E. A. (2002). A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* **160,** 1217–1229.

Bernatchez, L. (2001). The evolutionary history of brown trout (*Salmo trutta* L.) inferred from phylogeographic, nested clade, and mismatch analyses of mitochondrial DNA variation. *Evolution* **55,** 351–379. doi: 10.1111/j.0014-3820.2001.tb01300.x

Bernatchez, L., Renaut, S., Whiteley, A., Derome, N., Jeukens, J., Landry, L., Lu, G., Nolte, A., Ostbye, K., Rogers, S. & St-Cyr, J. (2010). On the origin of species: insights from the ecological genomics of lake whitefish. *Philosophical Transactions of the Royal Society B* **365,** 1783–1800. doi: 10.1098/rstb.2009.0274

Berrebi, P., Povz, M., Jesensek, D., Cattaneo-Berrebi, G. & Crivelli, A. J. (2000). The genetic diversity of native, stocked and hybrid populations of marble trout in the Soca River, Slovenia. *Heredity* **85,** 277–287. doi: 10.1046/j.1365-2540.2000.00753.x

Bettles, C. M., Docker, M. F., Dufour, B. & Heath, D. D. (2005). Hybridization dynamics between sympatric species of trout: loss of reproductive isolation. *Journal of Evolutionary Biology* **18,** 1220–1233. doi: 10.1111/j.1420-9101.2005.00935.x

Boni, C. (2000). Karst aquifers of the Central Apennines. *Hydrogeologie* **4,** 49–62.

Bourke, P., Magnan, P. & Rodriguez, M. A. (1997). Individual variations in habitat use and morphology in brook charr. *Journal of Fish Biology* **51,** 783–794. doi: 10.1111/j.1095-8649.1997.tb01999.x

Cairney, M., Taggart, J. B. & Hoyheim, B. (2000). Characterization of microsatellite and minisatellite loci in Atlantic salmon (*Salmo salar* L.) and cross-species amplification in other salmonids. *Molecular Ecology* **9,** 2175–2178. doi: 10.1046/j.1365-294X.2000.105312.x

Caputo, V., Giovannotti, M., Nisi Cerioni, P., Caniglia, M. L. & Splendiani, A. (2004). Genetic diversity of brown trout in central Italy. *Journal of Fish Biology* **65,** 403–418. doi: 10.1111/j.0022-1112.2004.00458.x

Carbone, A. (1965). *Vicalvi, Posta Fibreno, il Fibreno*. Frosinone: Tipografia dell'Abbazia di Casamari.

Charles, K., Guyomard, R., Hoyheim, B., Ombredane, D. & Baglinière, J.-L. (2005). Lack of genetic differentiation between anadromous and resident sympatric brown trout

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(*Salmo trutta*) in a Normandy population. *Aquatic Living Resources* **18,** 65–69. doi: 10.1051/alr:2005006

Cortey, M. & García-Marín, J. L. (2002). Evidence for phylogeographically informative sequence variation in the mitochondrial control region of Atlantic brown trout. *Journal of Fish Biology* **60,** 1058–1063. doi: 10.1111/j.1095-8649.2002.tb02429.x

Cortey, M., Pla, C. & García-Marín, J. L. (2004). Historical biogeography of Mediterranean trout. *Molecular Phylogenetics and Evolution* **33,** 831–844. doi: 10.1016/j.ympev.2004.08.012

D'Orsi, A. & Seminara, M. (2010). Dati preliminari di abbondanza e distribuzione di *Salmo fibreni* Zerunian & Gandolfi 1990 nel bacino di Posta Fibreno (Italia Centrale): primo utilizzo del visual census. *Studi Trentini di Scienze Naturali* **87,** 105–109.

Doyle, J. J. & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19,** 11–15.

Estoup, A., Presa, P., Krieg, F., Vaiman, D. & Guyomard, R. (1993). $(CT)_n$ and $(GT)_n$ microsatellites: a new class of genetic markers for *Salmo trutta* L. (brown trout). *Heredity* **71,** 488–496. doi: 10.1038/hdy.1993.167

Estoup, A., Rousset, F., Michalakis, Y., Cornuet, J.-M., Adriamanga, M. & Guyomard, R. (1998). Comparative analysis of microsatellite and allozyme markers: a case study investigating microgeographic differentiation in brown trout (*Salmo trutta*). *Molecular Ecology* **7,** 339–353. doi: 10.1046/j.1365-294X.1998.00362.x

Evanno, G., Regnaut, S. & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14,** 2611–2620. doi: 10.1111/j.1365-294X.2005.02553.x

Ferguson, A. & Mason, F. M. (1981). Allozyme evidence for reproductively isolated sympatric populations of brown trout *Salmo trutta* L. in Lough Melvin, Ireland. *Journal of Fish Biology* **18,** 629–642. doi: 10.1111/j.1095-8649.1981.tb03805.xù

Fraser, D. J., Weir, L. K., Bernatchez, L., Hansen, M. M. & Taylor, E. B. (2011). Extent and scale of local adaptation in salmonid fishes: review and meta-analysis. *Heredity* **106,** 404–420. doi: 10.1038/hdy.2010.167

Gandolfi, G., Zerunian, S., Torricelli, P. & Marconato, A. (1991). *I Pesci delle acque interne italiane*. Rome: Ministero dell'Ambiente, Istituto Poligrafico e Zecca dello Stato.

Garcia de Leaniz, C. & Verspoor, E. (1989). Natural hybridization between Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) in northern Spain. *Journal of Fish Biology* **34,** 41–46. doi: 10.1111/j.1095-8649.1989.tb02956.x

Gibertini, G., Ceccarelli, E. & Zerunian, S. (1990). Contributo alla conoscenza della riproduzione delle trote indigene nel Lago di Posta Fibreno (Lazio). *Rivista di Idrobiologia* **29,** 247–267.

Giuffra, E., Guyomard, R. & Forneris, G. (1996). Phylogenetic relationships and introgression patterns between incipient parapatric species of Italian brown trout (*Salmo trutta* L. complex). *Molecular Ecology* **5,** 207–220. doi: 10.1046/j.1365-294X.1996.00074.x

Hamilton, K. E., Ferguson, A., Taggart, J. B., Tomasson, T., Walker, A. & Fahy, E. (1989). Post-glacial colonization of brown trout, *Salmo trutta* L.: Ldh-5 as a phylogeographic marker locus. *Journal of Fish Biology* **35,** 651–664. doi: 10.1111/j.1095-8649.1989.tb03017.x

Hutchings, J. A. (2011). Old wine in new bottles: reaction norms in salmonid fishes. *Heredity* **106,** 421–437. doi: 10.1038/hdy.2010.166

Jonsson, B. & Jonsson, N. (2011). *Ecology of Atlantic Salmon and Brown Trout: Habitat as a Template for Life Histories*. Dordrecht: Springer.

Kahilainen, K. K., Østbye, K., Harrod, C., Shikano, T., Malinen, T. & Merilä, J. (2011). Species introduction promotes hybridization and introgression in *Coregonus*: is there sign of selection against hybrids? *Molecular Ecology* **20,** 3838–3855. doi: 10.1111/j.1365-294X.2011.05209.x

King, T. L., Eackles, M. S. & Letcher, B. H. (2005). Microsatellite DNA markers for the study of Atlantic salmon (*Salmo salar*) kinship, population structure, and mixed-fishery analyses. *Molecular Ecology Notes* **5,** 130–132. doi: 10.1111/j.1471-8286.2005.00860.x

Kottelat, M. & Freyhof, J. (2007). *Handbook of European Freshwater Fishes*. Cornol: Kottelat Berlin: Freyhof.

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Leary, R. F., Allendorf, F. W. & Sage, G. K. (1995). Hybridization and introgression between introduced and native fish. *American Fisheries Society Symposium* **15,** 91–101.

Machordom, A., Garcia-Marin, J. L., Sanz, N., Almodovar, A. & Pla, C. (1999). Allozyme diversity in brown trout (*Salmo trutta*) from Central Spain: genetic consequences of restocking. *Freshwater Biology* **41,** 707–717. doi: 10.1046/j.1365-2427.1999.00418.x

Matthews, M. A., Poole, W. R., Thompson, C. E., McKillen, J., Ferguson, A., Hindar, K. & Wheelan, K. F. (2000). Incidence of hybridization between Atlantic salmon, *Salmo salar* L., and brown trout, *Salmo trutta* L., in Ireland. *Fisheries Management and Ecology* **7,** 337–347. doi: 10.1046/j.1365-2400.2000.00208.x

McMeel, O. M., Hoey, E. M. & Ferguson, A. (2001). Partial nucleotide sequences, and routine typing by polymerase chain reaction-restriction fragment length polymorphism, of the brown trout (*Salmo trutta*) lactate dehydrogenase, LDH-C1*90 and*100 alleles. *Molecular Ecology* **10,** 29–34. doi: 10.1046/j.1365-294X.2001.01166.x

Meraner, A., Baric, S., Pelster, B. & Dalla Via, J. (2007). Trout (*Salmo trutta*) mitochondrial DNA polymorphism in the centre of the marble trout distribution area. *Hydrobiologia* **579,** 337–349. doi: 10.1007/s10750-006-0479-3

Meraner, A., Baric, S., Pelster, B. & Dalla Via, J. (2010). Microsatellite DNA data point to extensive but incomplete admixture in a marble and brown trout hybridisation zone. *Conservation Genetics* **11,** 985–998. doi: 10.1007/s10592-009-9942-9

Nonnis Marzano, F., Corradi, N., Papa, R., Tagliavini, J. & Gandolfi, G. (2003). Molecular evidence for introgression and loss of genetic variability in *Salmo* (*trutta*) *macrostigma* as a result of massive restocking of Apennine populations (Northern and Central Italy). *Environmental Biology of Fishes* **68,** 349–356. doi: 10.1023/B:EBFI.0000005762.81631.fa

Patarnello, T., Bargelloni, L., Caldara, F. & Colombo, L. (1994). Cytochrome b and 16S rRNA sequence variation in the *Salmo trutta* (Salmonidae, Teleostei) species complex. *Molecular Phylogenetics and Evolution* **3,** 69–74. doi: 10.1006/mpev.1994.1008

Poteaux, C., Bonhomme, F. & Berrebi, P. (1999). Microsatellite polymorphism and genetic impact of restocking in Mediterranean brown trout (*Salmo trutta* L.). *Heredity* **82,** 645–653. doi: 10.1046/j.1365-2540.1999.00519.x

Poulson, T. L. (2001). Adaptations of cave fishes with some comparisons to deep-sea fishes. *Environmental Biology of Fishes* **62,** 345–364. doi: 10.1023/A:1011893916855

Presa, P. & Guyomard, R. (1996). Conservation of microsatellites in three species of salmonids. *Journal of Fish Biology* **49,** 1326–1329. doi: 10.1111/j.1095-8649.1996.tb01800.x

Pritchard, J. K., Stephens, M. & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* **155,** 945–959.

Pujolar, J. M., Lucarda, A. N., Simonato, M. & Patarnello, T. (2011). Restricted gene flow at the micro- and macro-geographical scale in marble trout based on mtDNA and microsatellite polymorphism. *Frontiers in Zoology* **8,** 7. doi: 10.1186/1742-9994-8-7

Rasmussen, J. B., Robinson, M. D. & Heath, D. D. (2010). Ecological consequences of hybridization between native westslope cutthroat (*Oncorhynchus clarkii lewisi*) and introduced rainbow (*Oncorhynchus mykiss*) trout: effects on life history and habitat use. *Canadian Journal of Fisheries and Aquatic Sciences* **67,** 357–370. doi: 10.1139/F09-191

Salviani, I. (1554). *Aquatilium animalium historiae. Liber primus, cum eorundem formis aere excusis*. Rome: Ippolito Salviani.

Sell, J. & Spirkovski, Z. (2004). Mitochondrial DNA differentiation between two forms of trout *Salmo letnica*, endemic to the Balkan Lake Ohrid, reflects their reproductive isolation. *Molecular Ecology* **13,** 3633–3644. doi: 10.1111/j.1365-294X.2004.02362.x

Stearns, S. C. & Hendry, A. A. (2004). The salmonid contribution to key issues in evolution. In *Evolution Illuminated: Salmon and their Relatives* (Hendry, A. A. & Stearns, S. C., eds), pp. 3–19. Oxford: Oxford University Press.

Sušnik, S., Weiss, S., Odak, T., Delling, B., Treer, T. & Snoj, A. (2007). Reticulate evolution: ancient introgression of the Adriatic brown trout mtDNA in softmouth trout *Salmo obtusirostris* (Teleostei: Salmonidae). *Biological Journal of the Linnean Society* **90,** 139–152. doi: 10.1111/j.1095-8312.2007.00717.x

© 2013 The Authors

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28,** 2731–2739. doi: 10.1093/molbev/msr121

Zerunian, S. (2002). *Condannati all'estinzione? Biodiversità, biologia, minacce e strategie di conservazione dei Pesci d'acqua dolce indigeni in Italia*. Bologna: Edagricole.

Zerunian, S. & Gandolfi, G. (1986). Considerazioni preliminari sulle trote presenti nel bacino di Posta Fibreno (Lazio) (Pisces Salmonidae). *Acta Naturalia de l'Ateneo Parmense* **22,** 53–63.

Zerunian, S. & Gandolfi, G. (1990). *Salmo fibreni* n. sp. (Osteichthyes, Salmonidae) endemica nel bacino del Fibreno (Italia centrale). *Rivista di Idrobiologia* **29,** 521–532.

Zerunian, S., Lecce, A. & Gibertini, G. (1994). Osservazioni sulla riproduzione del Carpione del Fibreno (Osteichthyes, Salmonidae). In *Biologia dei salmonidi: tutela e gestione delle popolazioni indigene: Villa Cordellina, Montecchio Maggiore (VI),- Atti V Convegno nazionale AIIAD* (Associazione italiana ittiologi acque dolci), pp. 119–128. Vicenza: Provincia di Vicenza.

Electronic References

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N. & Bonhomme, F. (1996–2001). *GENETIX 4.02, logiciel sous Windows TM pour la génétique des populations*. Montpellier: Laboratoire Génome, Populations, Interactions, Université de Montpellier II. Available at http://kimura.univ-montp2.fr/genetix/

Freyhof, J. & Kottelat, M. (2008a). *Salmo fibreni*. *IUCN 2011. IUCN Red List of Threatened Species*. Version 2011.2. Available at www.iucnredlist.org (downloaded on 20 December 2011).

Freyhof, J. & Kottelat, M. (2008b). *Salmo cettii*. *IUCN 2011. IUCN Red List of Threatened Species*. Version 2011.2. Available at www.iucnredlist.org (downloaded on 20 December 2011).

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